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14. ABSTRACT   The objective of the research is to test the feasibility of a vitamin D biosensor based on a selective redox enzyme immobilized onto an electrode. In long-term, this will make it possible to create a technology akin to commercial glucose sensors for in-clinic use as part of routine examinations by both reducing the cost of testing vitamin D levels and reducing the time for testing to less than a few minutes. For this purpose, a synthetic gene for recombinant human CYP27B1 (the enzyme which is involved in vitamin D metabolism) was designed. The enzyme was expressed in *E. coli* and the activity of this enzyme was verified spectrophotometrically and using liquid chromatography-mass spectrometry (LC-MS/MS). Various synthetic redox mediators were tried and cobalt sepulchrate trichloride ($Co(sep)^{3+/2+}$) was found to enhance the direct electron transfer between electrode and redox center of CYP27B1. This is important for introducing a way to electrochemically measure 25(OH)D (circulating form of vitamin D in blood) levels.

15. SUBJECT TERMS
  Cytochrome P450, mediated electrochemistry, direct electron chemistry, vitamin D, 25(OH)D, electrochemical biosensors.

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1. Introduction

Vitamin D is crucial for the human body due to its role in calcium and bone metabolism. In addition, low blood levels of vitamin D levels have been associated with neuropsychiatric disorders.[1] Vitamin D blood tests, however, are expensive and the results are delayed. The purpose of this research is to test the feasibility of using an electrode modified with an enzyme that hydrolyzes circulating 25-hydroxyvitamin D (25(OH)D) into its biologically active form.[2] This, in the long-term, will make it possible to develop a clinical vitamin D sensor (similar to a commercial glucose sensor) that will reduce both the time and cost for testing. For this purpose, a synthetic gene for the expression of recombinant human cytochrome P450 (CYP27B1) in *E. coli* was designed, and solubilization strategies were developed to enable the purification of the active enzyme. We then explored the activity of the enzyme using non-natural redox mediators. This work provides a path forward for developing this enzyme into a biosensor for Vitamin D detection.

2. Keywords

Cytochrome P450, mediated electrochemistry, direct electron chemistry, vitamin D, 25(OH)D, electrochemical biosensors.

3. Overall Project Summary

3.1. DESIGN, EXPRESSION AND CHARACTERIZATION OF HUMAN CYP27B1, ADX AND ADR ENZYMES

3.1.1. Cloning, Expression and Purification of Human CYP27B1

Human CYP27B1 gene was codon optimized for expression and purification from *E. coli.* The N-terminal of CYP27B1 gene contains a 32-residue-long mitochondrial targeting sequence, which is truncated from the mature protein.[3] The mitochondrial targeting sequence was removed off and Ser-32 was mutated to Met which serves as the initiation codon. The gene was cloned into two different vectors pMAL-c4e (with and without maltose binding protein (MBP)) as a fusion to enhance expression, and pET20b with C-terminal *His-tag* (Figure 1).
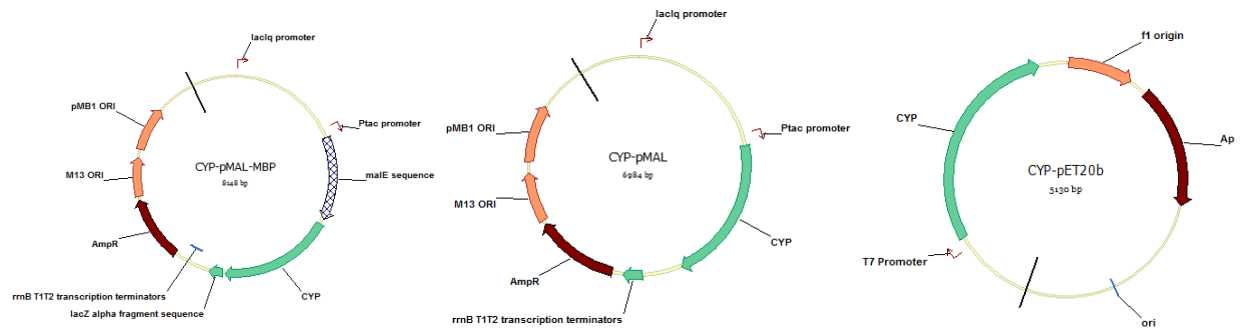
Figure 1: Plasmids containing the CYP27B1 gene. CYP-pMAL-MBP, CYP27B1 cloned in pMAL-c4e vector with MBP; CYP-PMAL, CYP27B1 cloned in pMAL-c4e vector without MBP; and CYP-pET20b, CYP27B1 cloned in pET20b vector.

All three plasmids were transformed into *E. coli* BL21 cells and expressed in the presence of the pGro7 plasmid encoding chaperons GroEL/ES.[4] After protein expression screening it was found that soluble protein was only obtained from CYP-pET20b. Both CYP-pMAL and CYP-pMAL-MBP enabled expression of CYP27B1, but the expressed protein was predominantly sent to inclusion bodies (Figure 2).
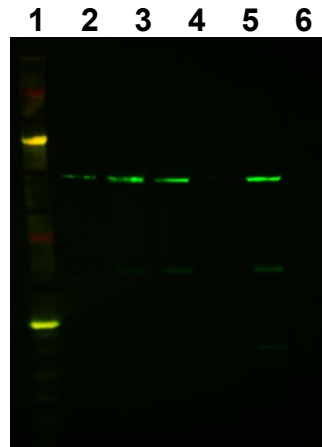

Figure 2: Western blot of CYP27B1 expression screening. Lane 1, 3 and 5 are CYP27B1 inclusion bodies from CYP-pET20b, CYP-pMAL and CYP-pMAL-MBP, respectively. Lane 2, 4 and 6 are CYP27B1 in solution from CYP-pET20b, CYP-pMAL and CYP-pMAL-MBP, respectively.

The cells containing both CYP-pET20b and pGro7 were inoculated in 5 mL TB broth containing 100 µg/mL ampicillin and 35 µg/mL chloramphenicol and incubated overnight at 37 C with shaking. The overnight culture was diluted into 2 x 1 L TB broth containing 100 µg/mL ampicillin and 35 µg/mL chloramphenicol. The cultures were incubated at 37 C until the cell density (OD_{600}) reached about 0.6. At this point, the induction of CYP27B1 and GroEL/ES transcription under the T7 promoter and the araB promoter were initiated by addition of IPTG and arabinose at final concentration of 0.5 mM and 200 µg/mL, respectively. δ-Aminolevulinic acid (ALA) was also supplemented at a final concentration of 1 mM. The cultures were incubated for another 20 h for the expression of CYP27B1 at 28 C.

The recombinant *E. coli.* cells were harvested by centrifugation and suspended in buffer A (20 mM Tris-HCl, pH 7.4 containing 1% CHAPS, 200 mM NaCl, 0.1 mM PMSF, and 20% glycerol). Cells were disrupted by sonication in the presence of HALT protease inhibitor for 20 min in the presence of ice. Cell debris was removed by centrifugation and the resultant supernatant was applied to a Ni affinity column equilibrated with buffer A. The column was washed with 5 column volumes of buffer A. CYP27B1 was eluted with 100 mM imidazole in buffer A (buffer B). Eluted fractions were checked for purity (Figure 3), and the pure fractions were collected and dialyzed in buffer C (50 mM Tris-HCl, pH 7.4 containing 0.1% CHAPS, 200 mM NaCl, 0.1 mM PMSF, and 20% glycerol) and stored at -80 C.
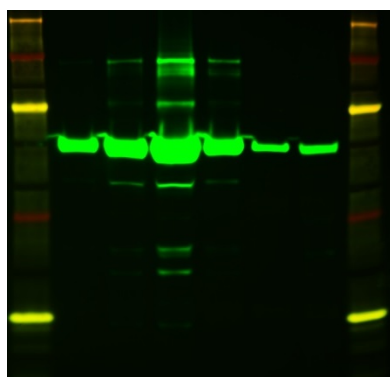

Figure 3: Western blot of pure CYP27B1 fractions.

3.1.2. Cloning, Expression and Purification of Human Adrenodoxin (Adx) and NADPH-Adrenodoxin Reductase (Adr)

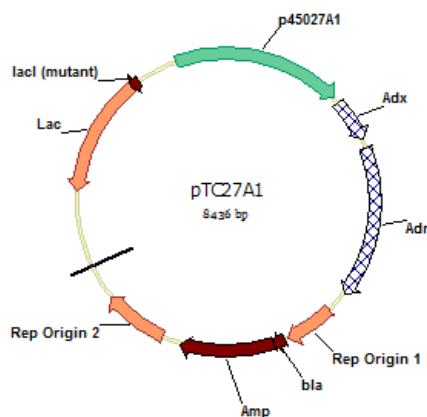Human adrenodoxin (Adx), and NADPH-adrenodoxin reductase (Adr) genes were amplified from the pTC27A1 plasmid (Figure 4).[5]

Figure 4: Vector pTC27A1 containing human Adx, Adr and p45027A1.

Adx and Adr were cloned into pMAL-c4E separately, creating pMAL-ADX (Figure 5) and pMAL-ADR (Figure 6), respectively. Both Adx and Adr were also cloned together with CYP27B1, creating pCXR (Figure 7).
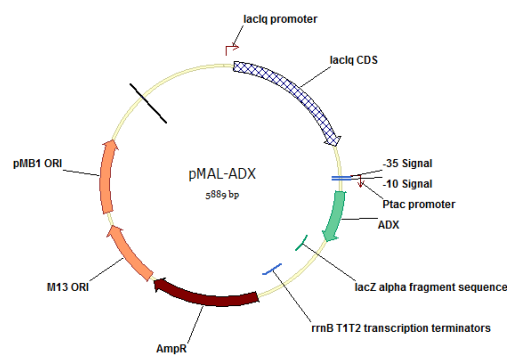
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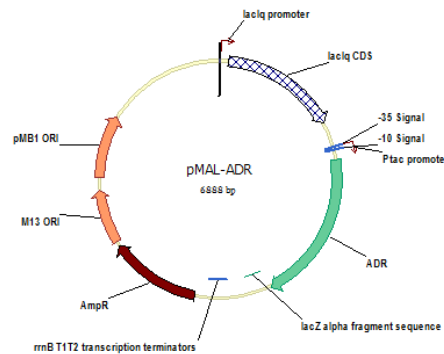**Figure 5:** Vector pMAL-ADX containing human Adx.

Figure 6: Vector pMAL-ADR containing human Adr.
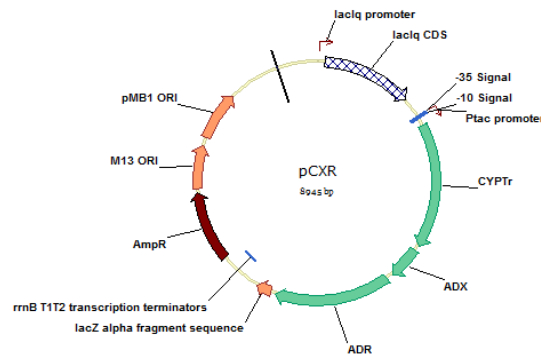

Figure 7: Vector pCXR containing CYPTr, Adx and Adr.

All three plasmids, pMAL-ADX, pMAL-ADR and pCXR, were transformed into *E. Coli* BL21 and BL21/pGro7 competent cells and grown in 5 mL TB media supplemented with 0.4% glycerol (v/v), 2 mg/mL bactopeptone, 1 mM NaCl, 1mM thiamine, a mixture of trace elements (50 μM ferric citrate, 1mM $MgCl_2$, 2.5 mM $(NH_4)_2SO_4$), 100 μg of Amp/mL and 35 μg of Cam/mL at 37 C till OD_{600} ~ 0.6 and were induced with 1 mM IPTG, 1 mM δ-aminolevulinic acid (ALA), 200 μg of Arabinose/mL, 2μg of Hemin/mL and grown at 28 C for ~ 24 h. Cells were harvested with centrifugation, suspended in 0.5 mL Tris-HCl buffer, pH 7.4 and sonicated. Both cell debris and supernatants were subjected to SDS-PAGE and western blotting was performed to determine protein expression (Figure 8).
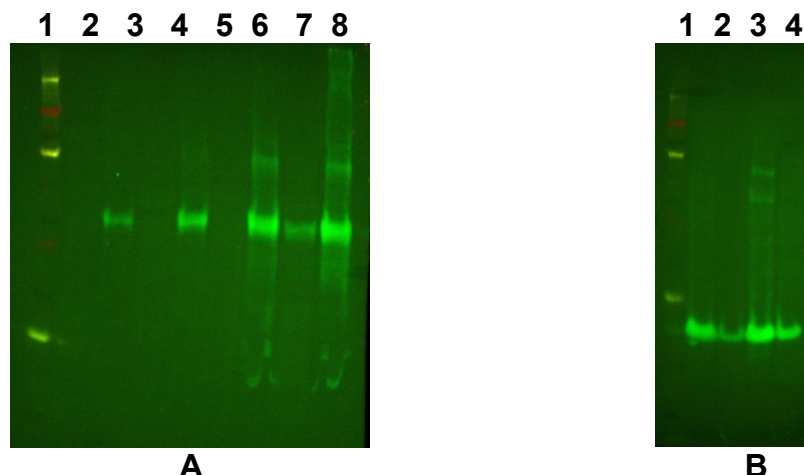
Figure 8: A) 1. pCXR/BL21 in solution, 2. pCXR/BL21 in insoluble fractions, 3. pCXR/BL21/pGro7 in solution, 4. pCXR/BL21/pGro7 in insoluble fraction, 5. Adr/BL21 in solution, 6. Adr/ BL21 in insoluble fraction, 7. Adr/BL21/pGro7 in solution, 8. Adr/BL21/pGro7 in insoluble fraction. B) 1. Adx/BL21 in solution, 2. Adx/BL21 in insoluble fraction, 3. Adx/BL21/pGro7 in solution, and 4. Adx/BL21/pGro7 in insoluble fraction.

All three plasmids, pCXR, pMAL-Adr and pMAL-Adx, were found to enable protein expression, but in the case of pCXR the proteins were found in inclusion bodies. Therefore, we decided to purify Adr and Adx separately from BL21/pGRO7 cells.

2 L cultures of pMAL-Adr/BL21/pGro7 and pMAL-Adx/BL21/pGro7 were grown in the above mentioned media. Cells were harvested with centrifugation, suspended in 40 mL buffer D (100 mM Tris-HCl, pH 7.4 containing 1% CHAPS, 500 mM NaCl, 0.5 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, and 20% glycerol) and disrupted by sonication (20 min, 5 sec On, 10 sec Off). Cell debris was removed by centrifugation, and supernatant was loaded into pre-equilibrated Ni column with buffer D and eluted with 100 mM imidazole in buffer D (buffer E). The fractions were run in SDS-PAGE gel and pure fractions were collected for dialysis into buffer C.

3.1.3. Spectrophotometric activity assay for CYP27B1

The hydroxylation of 25(OH)D to 1,25(OH)$_2$D is catalyzed by CYP27B1 in the presence of NADPH as a cofactor. NADPH is oxidized to NADP by transferring electrons to CYP27B1 via Adx and Adr for required hydroxylation.[6-8] So, the rate of 25(OH)D hydroxylation by CYP27B1 can be monitored spectrophotometrically by monitoring the rate of NADPH oxidation to NADP at 340 nm.

Pure CYP27B1 was used for the activity assay at room temperature in 50 mM Tris-HCl buffer, pH 7.4 containing 0.1 mM EDTA, 0.1% CHAPS and 0.2% glycerol. The reactions were followed by the oxidation of 192 µM NADPH, in the presence of 2 µM Adx, 0.3 µM Adr, 30 µM 25(OH)D and 125 µM CYP27B1. As a control, NADPH oxidation was

monitored in absence of CYP27B1, where 25(OH)D, Adx and Adr were added to the reaction mixture. No NADPH oxidation was observed (Figure 9, green line); confirming NADPH oxidation is independent of Adx and Adr. NADPH oxidation was, also, monitored in the absence of 25(OH)D, where Adx, Adr and CYP27B1 were added to the reaction mixture. NADPH oxidation was observed independent of 25(OH)D addition (Figure 9, red line). This is due to the formation of superoxide or peroxide independent of substrate during the electron transfer from NADPH to CYP27B1, resulting in substrate independent NADPH oxidation. When substrate was added to the reaction, the initial rate of NADPH oxidation increased two-fold (Figure 9, blue line). Therefore the rate of 25(OH)D hydroxylation by CYP27B1, 0.8 min^{-1}, was calculated by subtracting the background rate of NADPH oxidation in the absence of 25(OH)D.
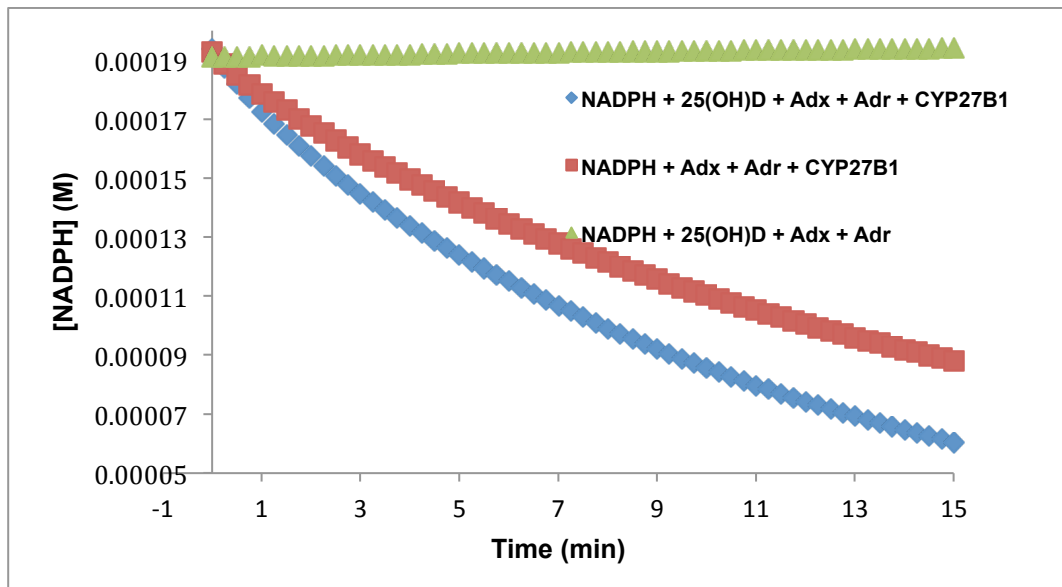

Figure 9: CYP27B1 activity assay results. There is significant NADPH oxidation in the absence of 25(OH)D, however the NADPH oxidation increases with the presence of 25(OH)D indicating the presence of active enzyme.

3.1.4. Analysis of CYP27B1 Activity via LC-MS/MS

Although spectrophotometric NADPH oxidation assay provides a way to determine the activity of CYP27B1, LC-MS/MS (Liquid Chromatography-Mass Spectrometry) should be used in order to determine the amount of 1,25(OH)$_2$D and other possible metabolites produced by CYP27B1.

For the assay, purified CYP27B1 was used in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA, 0.1% CHAPS, and 0.2% glycerol. The reactions consisted of 192 µM NADPH, 20 µM ADX, 0.45 µM ADR, 5-15 µM 25(OH)D and 100-200 µM CYP27B1. After preincubation at 37 C for 2 minutes, the reactions were initiated with the addition of NADPH. The reactions were stopped by addition of acetonitrile after 2-10 minutes and the samples were submitted to Irving Institute of Columbia University Medical Campus for analysis with LC-MS/MS. Liquid chromatography results show production of 1,25(OH)$_2$D and also formation of a (OH)$_2$D isomer, which has a retention time similar but not identical to 24,25(OH)$_2$D. Various experiments were conducted to investigate the activity of
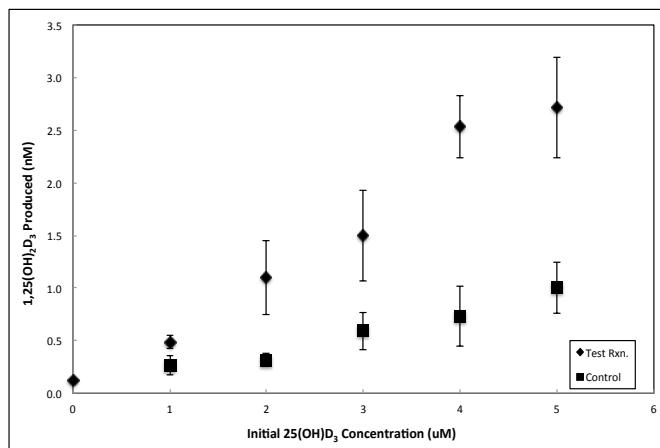
CYP27B1.

Figure 10: Concentration of 1,25(OH)$_2$D produced as a function of initial 25(OH)D concentration for the complete reaction (test experiment) and control (no CYP27B1) experiment.

Figure 10 shows the results for the production of 1,25(OH)$_2$D as a function of initial 25(OH)D present in the assay. Concentration of 1,25(OH)$_2$D increased as the initial concentration of the reactant in the assay was increased. As a control, experiments were also conducted without CYP27B1 in the assay. With the exclusion of the enzyme in the system, changes in the product concentrations were considerably smaller, which might be due to the impurities in the 25(OH)D sample.
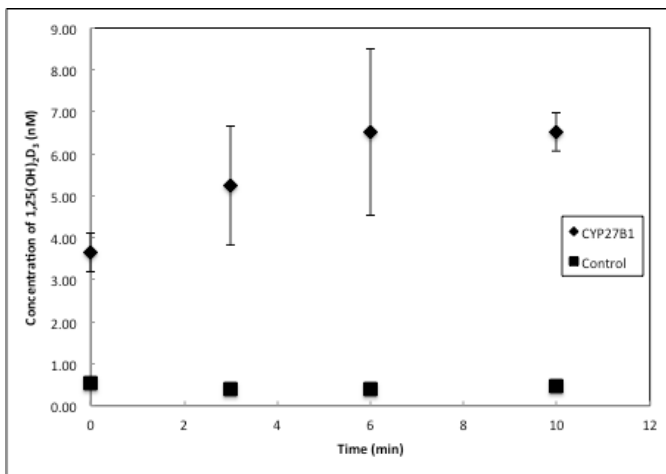

Figure 11: Results obtained with LC-MS/MS. Concentration of 1,25(OH)$_2$D produced over time.
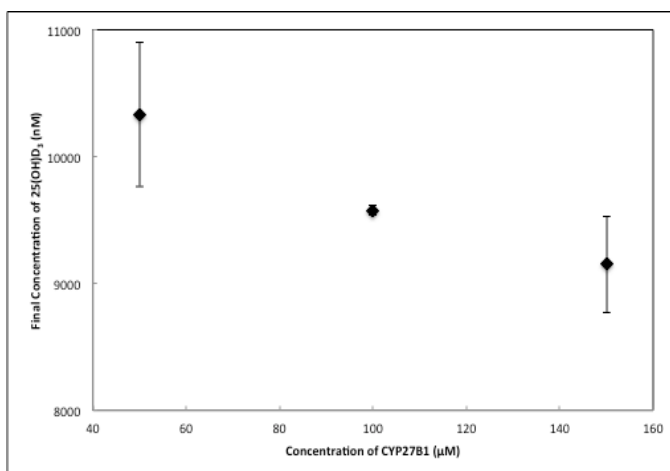
Figure 12: Final concentration of 25(OH)D left in the reaction as a function of concentration of CYP27B1 in the assay.

In the experiment described in Figure 11, the enzymatic reaction is stopped at different times in order to check production of 1,25(OH)$_2$D over time. Product formation starts with a value of 3.5 nM, showing a rapid production of 1,25(OH)$_2$D, and reaches to steady state with a value of 6.5 nM after 6 minutes. Although the yield for 1,25(OH)$_2$D$_3$ is low, 25(OH)D is consumed by CYP27B1 over time. In an experiment to demonstrate this (Figure 12), we altered the concentration of CYP27B1 in the reaction mixture and measured the final concentration of 25(OH)D not consumed by the reaction. We have observed that consumption of the substrate increased when the amount of CYP27B1 was increased in the assay, which is most relevant to biosensor development since the biosensor is designed to measure changes in 25(OH)D concentration, not those in the products. Overall, these results suggest that recombinantly expressed CYP27B1 is successful in converting 25(OH)D into 1,25(OH)$_2$D although the yields for 1,25(OH)$_2$D are low.

3.2. ELECTROCHEMICAL MEASUREMENT OF 25(OH)D LEVELS

3.2.1. Investigation of Mediated Electron Transfer with Phenosafranine and Safranin T

Mediated electron transfer using synthetic redox mediators is an alternative to the natural electron transfer systems in P450s, which use NADPH as electron supplier and Adx and Adr as electron transfer enzymes. Synthetic redox mediators transfer electrons rapidly from the electrode to the active redox site of the enzyme.

We have initially explored two synthetic redox mediators, phenosafranine (PSF) and safranin T (SAF). These organic mediators contain two amine groups that can interact with hydrophilic residues of enzyme and these residues may play an important role in electron transfer by creating a docking surface.[9] In Figure 13, the oxidation potential of PSF has been investigated with cyclic voltammetry, using Au as the working electrode, Ag/AgCl as the reference electrode and Pt as the counter electrode. It has been found that PSF has an oxidation potential of -0.242 V (vs. SHE) and this value is close to the literature value of -0.252 V (vs. SHE).[10]
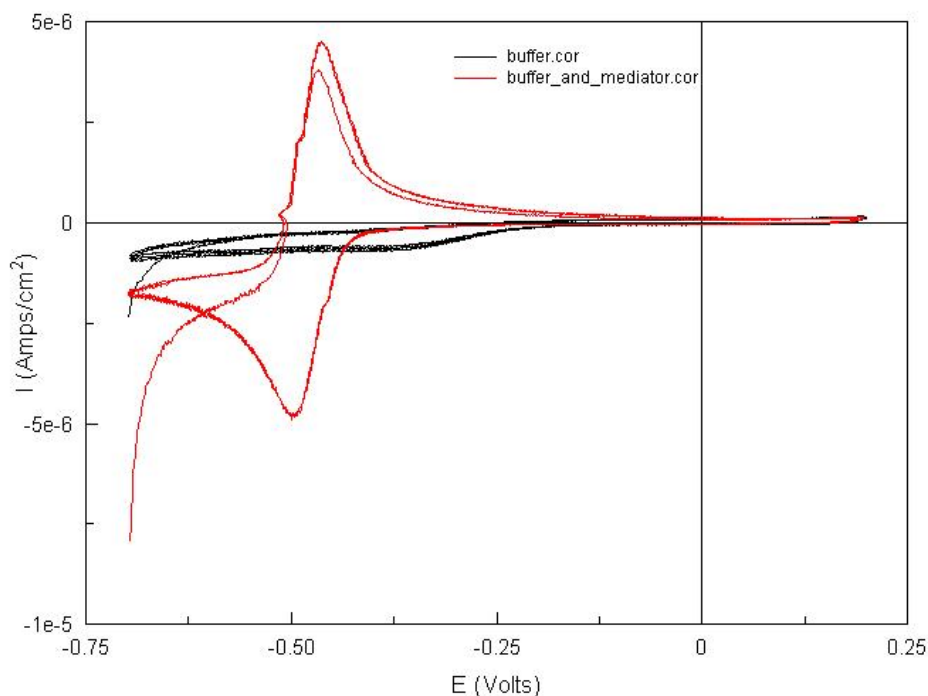
Figure 13: Cyclic voltammetry (CV) for the characterization of the oxidation potential of PSF. The black curve shows the CV for the electrolyte solution (0.1 M PBS, pH 7.4), whereas the red curve represents the case where 0.6 mM PSF is solubilized in the same electrolyte solution. Potential values are with respect to the Ag/AgCl reference electrode.

Following this experiment, varying amounts of CYP and the substrate (25(OH)D) were added to the PSF containing solutions in order to observe if mediation occurs. In this case, the enzyme, mediator and substrate are in solution, meaning the system is homogenous. In homogeneous mediation, the mediator first changes its redox state at the electrode. This mediator diffuses from the electrode and transfers electrons to the enzyme, and in turn, this enzyme reacts with the substrate to produce the desired product.[11]

Cyclic voltammetry was utilized to observe the changes in the oxidation/reduction peaks. Figure 14 shows the case where substrate (25(OH)D) concentration is kept constant at 5 µM, and varying amounts of CYP27B1 (0-10 µM) are added into the solution, and a change in the peak current is plotted as a function of concentration of CYP27B1. When the amount of enzyme in the system was increased, the peak where PSF oxidation/reduction peaks are observed is altered; meaning the amount of mediator oxidized/reduced is changed. This is in agreement with the proposed homogeneous mediation scheme given above. Similar results can be seen in Figure 15, where this time the CYP27B1 concentration is kept constant and 25(OH)D concentration is changed. In Figure 12, the change in the current for different modes of operation (oxidation vs reduction peaks) is plotted as a function of the concentration of 25(OH)D.
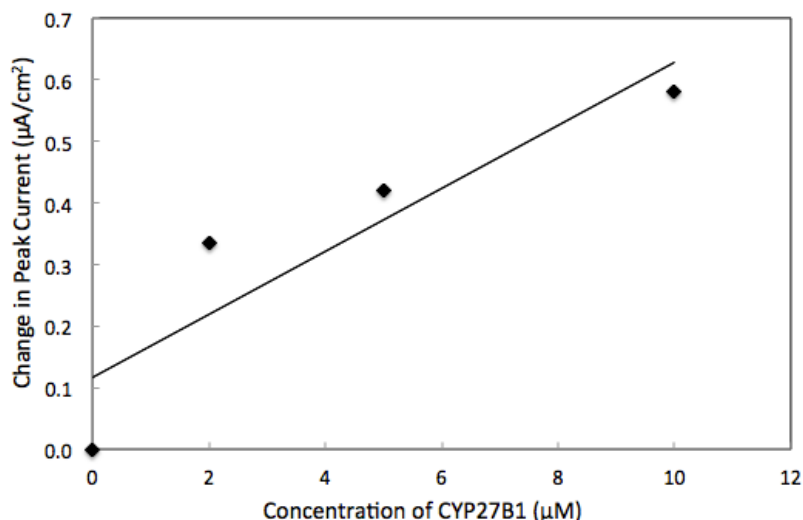
Figure 14: Change in peak currents as a function of concentration of CYP27B1. Experiments were made in the presence of 2000 ng/mL 25(OH)D. The electrolyte solution is 0.1 M PBS (pH 7.4) that contains 0.3 mM mediator (phenosafranine).
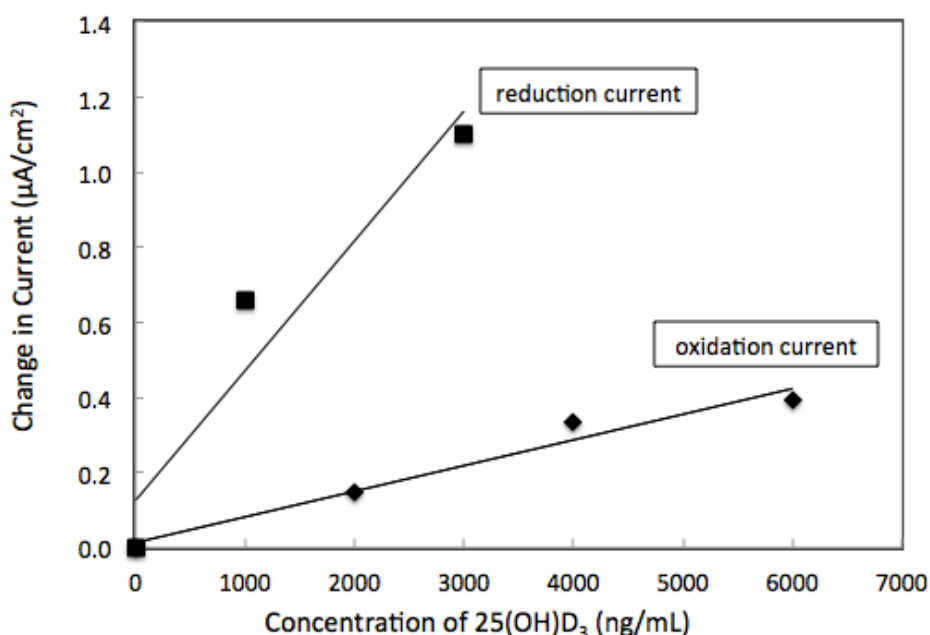

Figure 15: Change in reduction and oxidation currents as a function of concentration of $25(OH)D_3$. Reduction current experiments were made in the presence of 2 µM CYP27B1 whereas oxidation experiments were made in the presence of 5 µM CYP27B1. The electrolyte solution is 0.1 M PBS (pH 7.4) that contains 0.3 mM mediator (phenosafranine).

Similar experiments were also performed for the other mediator, safranin T. In control experiments, where only mediator and substrate added (in order to see if the mediator reacts with the substrate), it has been observed that safranin T reacts directly with the substrate, limiting its practical utility. In contrast, the PSF does not react directly with the substrate 25(OH)D.

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3.2.2. Investigation of Direct Electron Transfer with Cobalt Sepulchrate Trichloride

Electrochemical experiments performed in bulk electrolyte systems and with phenosafranine did not yield reproducible and reliable results, likely due either to incomplete or insufficient mixing of substrate within the system (bulk electrolyte volume was at least 100 times larger than the volume of substrate added to the system) or due to the diffusion of materials off of the working electrode and into the bulk electrolyte system. To overcome these limitations, a salt-bridge cell, wherein the electrodes were placed flush against a piece of filter paper saturated in electrolyte, was constructed. This, in turn, mitigated concerns regarding the diffusion of materials off of the working electrode and allowed for the introduction of substrate directly to the enzyme-modified working electrode. Figure 16 provides an illustration of this cell design. In addition, cobalt sepulchrate trichloride, a non-native redox mediator, was synthesized and used in this new setup. Control experiments were performed to ensure that effective charge transfer between electrodes was possible before applying this system to the development of a vitamin D sensor.
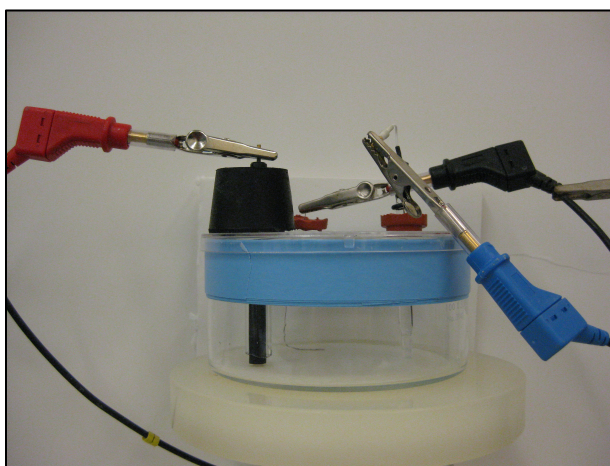

Figure 16: Salt-bridge cell utilized for the collection of electrochemical data.

Since cobalt sepulchrate trichloride ($Co(sep)Cl_3$) is not available commercially, it was synthesized chemically as follows: To a stirring suspension of Li_2CO_3 (2.3g, excess) and ±$[Co(en)_3]Cl_3$ (0.9g, 2.60 mmol) in deionized water (20 mL) were added separate solutions of 37% CH_2O (50 mL, *aq*) and 28% NH_4OH (50 mL, *aq*) via addition funnel over a period of roughly 50 minutes. Once the addition was complete, the reaction mixture was stirred for an additional 30 minutes, at which point unreacted Li_2CO_3 was filtered off. [12]

Isolation of $Co(sep)Cl_3$ from the reaction mixture was performed using the following procedure. A solution of $Na[S_2CNEt_2]\cdot3H_2O$ (2.5g, 11.1 mmol) in deionized water (50 mL) was prepared and then added to the reaction mixture and stirred for approximately 30 minutes; red and green precipitates crashed out of solution. The solid was isolated and washed with 20:80 CH_2Cl_2:hexanes (3x50 mL) to isolate the desired red crystals of $[Co(sep)][S_2CNEt_2]$ (77.4% yield). These crystals were then suspended in acetonitrile (10 mL, neat) and quenched with concentrated HCl, causing the red crystals to decompose rapidly, resulting in a pale-yellow solution. This solution was warmed for approximately 20 minutes, allowed to cool, and the yellow crystals of $Co(sep)Cl_3$ were collected via vacuum filtration (76.3% yield). [13]

The electrodes are prepared using the following procedure: The glassy carbon working electrode (GCE) was activated by mechanical polishing with an aqueous slurry of 0.5 µm alumina for one minute using a Buehler rotary polisher. The GCE was then sequentially rinsed with methanol and DI H_2O and sonicated in DI H_2O for five minutes to remove residual polishing material. This process was then repeated a second time; instead of sonicating in DI H_2O, the GCE was sonicated in absolute ethanol for five minutes. The GCE was then rinsed with DI H_2O and dried under a gentle nitrogen flow for approximately 5 minutes.

Nafion-117 stock solution was diluted to 1% w/v using 2-propanol and was pH adjusted with Tris-buffer, yielding a 1% solution (pH 7.395) of **A**. This was followed by the preparation of a 1mM solution of Co(sep)Cl$_3$ in 0.1 M PBS (pH 7.4), yielding **B**. Purified CYP27B1 was used without any modification, yielding **C**. The following electrode coating procedure was used: 5µL of **A** was drop-cast onto the clean/dry GCE, followed sequentially by 5µL of **B** and 5µL of **C**. The electrode was then dried under a gentle flow of nitrogen for approximately 10 minutes.

All electrochemical measurements were performed at room temperature (~25°C) in the presence of atmospheric oxygen using 0.1 M PBS (pH 7.4) as electrolyte. In addition to the modified GCE, a Ag/AgCl (3M NaCl reservoir) reference electrode and a platinum wire counter electrode were used for all experiments. Cyclic voltammetric (CV) analyses were performed on an EG&G PAR Potentiostat/Galvanostat model 273A under the control of CorrWare software; CorrView software was used for the visualization of results. All CV experiments were conducted at a scan rate of 100 mV/s and across a variable range of potentials. Square-wave voltammetric (SWV) analyses were carried out on a µAutolab potentiostat at 15 Hz, 4.05 mV step height, and 25 mV amplitude on a potential range from 0 to -800 mV.

Cyclic voltammograms of the GCE/Nafion/Co(sep)/CYP27B1 system demonstrated two cathodic peaks, one indicative of the Co$^{III/II}$ transition and the other occurring at the approximate redox potential of CYP27B1.[2] Both peaks were responsive upon the addition of substrate; however, in the absence of Co(sep)$^{3+}$, the peak corresponding to the Fe$^{III/II}$ transition of the heme group was either not observed or poorly defined. CVs of GCE modified only with Nafion did not exhibit electrochemical activity as expected. Figure 17 summarizes the various behaviors observed in CV analyses, and serves to suggest that the visualization of direct electron transfer to the heme group of the enzyme was enhanced by the presence of Co(sep)$^{3+}$.
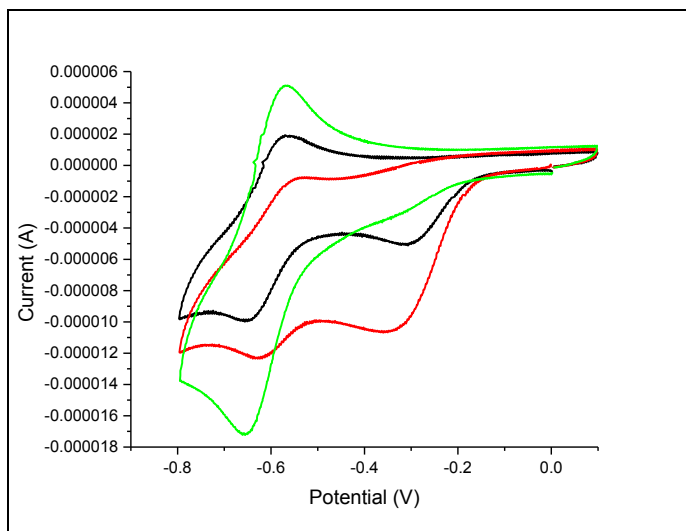
Figure 17: Representative cyclic voltammograms showing modified working electrode behavior of (green trace) GCE/Nafion/Co(sep)$^{3+}$, (black trace) GCE/Nafion/Co(sep)$^{3+}$/CYP27B1, and (red trace) GCE/Nafion/Co(sep)$^{3+}$/CYP27B1 following the addition of 10μL of 205.1 ng/ml substrate to the working electrode.

Square-wave voltammetric analyses were performed to obtain higher resolution of Fe$^{II/III}$ behavior both before and following the addition of substrate. Figure 18 provides a summary of the typical behavior observed. In brief, in the absence of an enzyme concentration on the working electrode, adverse current changes were observed, suggesting that Co(sep)$^{3+}$ itself is not capable of transferring electrons to the substrate, as expected. However, when the working electrode was modified with both Co(sep)$^{3+}$ and CYP27B1, peak current growth at the Fe$^{II/III}$ signal was observed while peak current decay at the Co$^{III/II}$ signal was observed. This evidence suggests that the enzyme is responsive to 25(OH)D concentrations; the Co$^{III/II}$ peak current decay is explained by an examination of the forward and reverse spectra obtained both before and following substrate addition (data not shown), or by the CV shown in Figure 16 where the anodic peak corresponding to the Co(sep)$^{3+}$ complex always exhibits a downward shift following the addition of 25(OH)D.
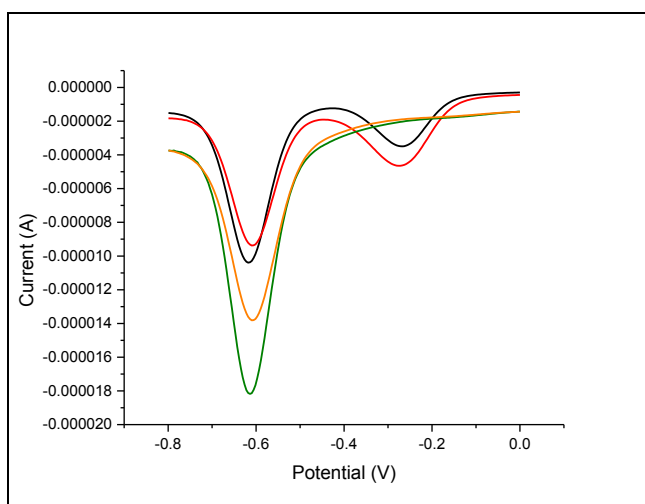

Figure 18: Representative square-wave voltammograms of the working electrode modified with (green trace) Nafion/Co(sep)$^{3+}$, (orange trace) Nafion/Co(sep)$^{3+}$ in the

presence of 10 µL of 25.6 ng/ml substrate, (black trace) Nafion/Co(sep)$^{3+}$/CYP27B1, and (red trace) Nafion/Co(sep)$^{3+}$/CYP27B1 in the presence of 10µL of 25.6 ng/ml substrate.
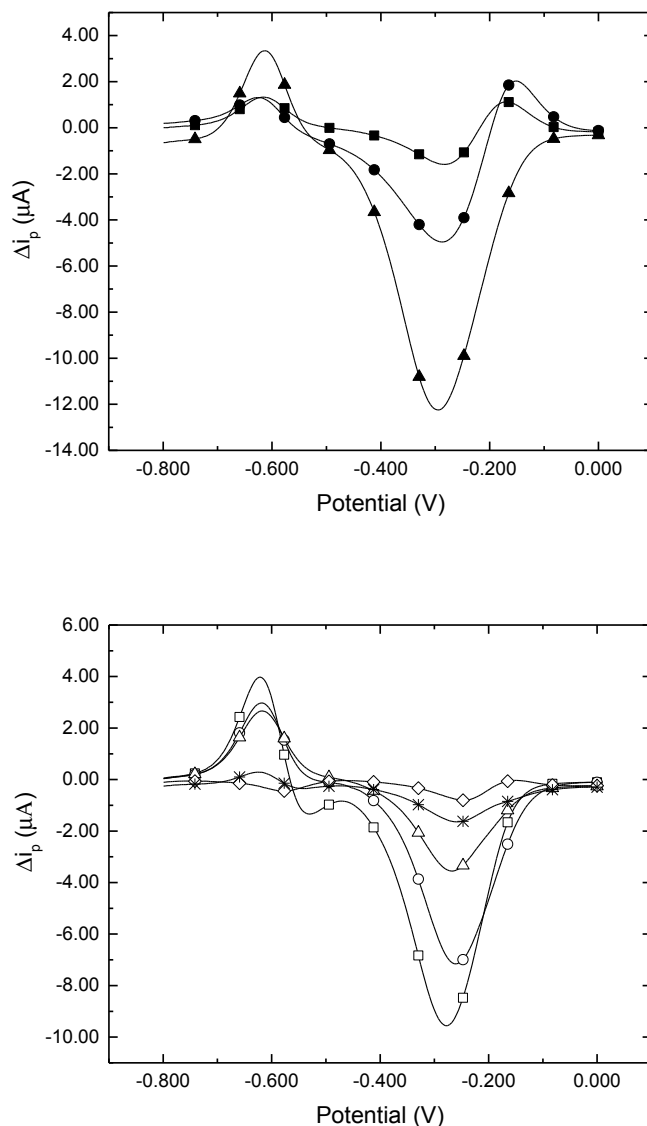

Figure 18: Plots derived from square-wave voltammograms, showing the difference in peak current (peak current following addition of substrate minus baseline peak current) vs. potential for a series of 25(OH)D concentrations within the physiological range. 10µL of substrate was added to the working electrode in each case. ***Top:*** (■) 25.6 ng/mL, (●) 51.3 ng/mL, (▲) 76.9 ng/mL. ***Bottom:*** (□) 102.5 ng/mL, (○) 128.2 ng/mL, (Δ) 153.9 ng/mL, (◊) 179.5 ng/mL, (*) 205.1 ng/mL.

Figure 18 illustrates the proof of function of our design – a plot of change in current vs. potential for a series of 25(OH)D concentrations, all within the physiological range, serves to verify that different substrate concentrations result in distinguishable current changes. As the concentration of substrate increases in the lower physiological range, peak current monotonically increases as expected. However, in the upper physiological

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range (larger than 76.9 ng/ml), peak current monotonically decreased as the substrate concentration was increased except the upper limit.

4. Key Research Accomplishments

1. A synthetic gene for recombinant human CYP27B1 was designed for *E. coli*.
2. CYP27B1 was expressed and purified.
3. Recombinant bovine ferredoxin (ADX) and ferredoxin reductase (ADR) were expressed and purified.
4. The activity of CYP27B1 was verified via spectrophotometric NADPH oxidation assay and using liquid chromatography-mass spectrometry (LC-MS/MS).
5. A cobalt sepulchrate trichloride and CYP27B1 modified glassy carbon electrode was found to be successful for amperometric detection of 25-hydroxyvitamin D (25(OH)D) in a salt-bridge cell with reproducible results.
6. The enzyme modified electrode was found to be successful in detecting 25(OH)D in buffer within physiological range (5-200 ng/ml).

5. Conclusion

In this research, the feasibility of a vitamin D biosensor based on a selective redox enzyme immobilized onto an electrode was investigated. A synthetic recombinant enzyme, CYP27B1, was expressed in *E. coli*. This enzyme is responsible for hydroxylation of 25-hydroxyvitamin D (25(OH)D, circulating form of vitamin D in blood) into its active form. This enzyme is membrane associated, and therefore solubilization strategies were developed. The activity of the enzyme was verified both spectrophotometrically using NADPH oxidation assay and using liquid chromatography-mass spectrometry (LC-MS/MS). For these assays, recombinant bovine ferredoxin (ADX) and ferredoxin reductase (ADR) enzymes were expressed and purified. Finally, a method was established for electrochemical measurement of 25(OH)D levels by introducing a non-natural redox mediator. Various strategies with three different synthetic redox mediators were explored and a glassy carbon electrode modified with cobalt sepulchrate trichloride and human CYP27B1 enzyme is found to be capable of responding to the circulating form of vitamin D, 25(OH)D, suggesting we can detect the activity of the enzyme electrochemically. Using different electrochemical techniques, this enzyme electrode was found to be successful in detection of 25(OH)D in buffer within physiological range (5-200 ng/ml). For future work, significant optimization of the electrode construction and mode of detection is required. Furthermore, detection of 25(OH)D in blood samples will be an important next step to understand the effects of interferents. These promising results chart a path forward for the development of a vitamin D biosensor akin to commercial glucose sensor that will reduce both the time for testing and the cost of 25(OH)D assay and that can be utilized in clinically.

6. Publications, Abstracts, and Presentations

Ozbakir, H. (speaker), Sambade, D., Majumdar, S., Banta, S. A. and West, A.C. "Towards the Development of an Electrochemical Biosensor for the Detection of Vitamin D" AIChE Annual Meeting, Atlanta, GA, November 2014.

Ozbakir, H.F., Sambade, D.A., Mujimdar, S., Linday, L. Banta, S.A. and West, A.C. "Detection of 25-hydroxyvitamin D with an Enzyme Modified Electrode", *In Preparation*.

7. Inventions, Patents, and Licenses

West, A., Banta, S., Sambade, D., Ozbakir, H. and Majumdar, S. "Methods and Systems for Point-of-Care Measurement of Vitamin D Levels" Application No: 62/054,118, Filing Date: September 23, 2014.

8. Reportable Outcomes

1. Manuscript in preparation.

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